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Chemical Category
BENZENE, 1,1'-METHYLENEBIS[4-ISOCYANATO 4,4-METH* (101-68-8)

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OFFICE OF TOXIC SUBSTANCES

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Name of Chemical Substance:	benzene 1,1` –methylenebis[4-isocyanato
Common name:	4,4` –methylene diphenyl dissocyanate monomeric MDI
Chemical Abstracts Service Number:	101-68-8
Abbreviation:	4,4` -MDI
Authors:	A.L. Kennedy and W.E. Brown

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Biochemical and Histoautoradiographic Characterization of the Distribution of Radioactivity Following Exposure to 14C-MDI Aerosol

FINAL REPORT

Project ID : 103-AM-MTX

11321

1 October 1998

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SUMMARY

Inhalation exposure to diisocyanates can result in a variety of airway diseases. In the case of polymeric diphenylmethane diisocyanate (MDI) additional concern has been expressed due to the detection, in animal models, of lung tumors following inhalation of aerosols after chronic exposure at high concentrations. To begin to investigate the etiology of this possible disease process, this study was performed with monomeric 4,4'-14C-MDI which allowed the analysis of the distribution of the compound at the tissue, cell and macromolecular levels. Rats were exposed to 14C-MDI aerosols at target concentrations of 0.05 mg/m3 and 0.4 mg/m3 for four hours. All tissues examined showed detectable quantities of radioactivity, with the airways, gastrointestinal system and blood having the highest levels. The concentration of radioactivity in the bloodstream after 4 hours of exposure increased with increasing dose. The majority (74-79%) of the label associated with the blood was recovered in the plasma, and of this, 93-100% of the 14C existed in the form of biomolecular conjugates. Thus, the circulating form of the compound appeared to be quite similar to the distributions found following 14C-toluene diisocyanate vapor exposure (Kennedy and Brown, 1994). For both diisocyanates it appeared that conjugation with proteins was the predominant reaction and that free amine or other low molecular weight adducts or metabolites were not the predominant, *in vivo* reaction products under the conditions tested.

To complement the biochemical distributions, histoautoradiographic analysis of airway sections from 14C-MDI-exposed rats showed predominantly surface labeling but no significant airway alteration. An additional group of animals was exposed to unlabeled 4,4'-MDI at a higher aerosol concentration (6 mg/m3) for four hours to examine potential concentration related damage. Even at the highest MDI aerosol concentration, no changes in airway morphology were observed when compared to the unexposed control group.

INTRODUCTION

The *in vivo* reactivities of a variety of isocyanates have been investigated through the use of radiolabeled pure compounds (Kennedy, 1990). These studies have shown that despite the high degree of isocyanate reactivity, selective reactions can be

detected in the airway and bloodstream. Only slight differences in these reactions were observed for the different isocyanates tested.

Diphenylmethane diisocyanate (MDI) is one particular isocyanate in which there is heightened current interest. Vapor exposure to this compound is believed to be minimal due to its low vapor pressure. Therefore, predominant routes of MDI exposure are believed to be dermal and inhalation of aerosol particles in workplace applications. Aerosol inhalation distinguishes MDI from some of the other isocyanates which have been studied using radiolabelled forms and this route of exposure may affect *in vivo* reactivity and fate. Clear differences between TDI and MDI, for instance, have been observed during carcinogenicity testing. In a long term bioassay on MDI, a small number of lung tumors were detected in the 6 mg/m3 exposure group (Kuzel *et al.*, 1990). The tumor development may be due to an inflammatory response rather than as the direct action of the MDI itself. It is therefore important to understand the etiology of this disease process and the role of reactivity of the MDI itself may or may not play.

Very little is known about the reactivity and fate of aerosolized MDI *in vivo*. A study performed by Saclay (1977) using 14C-MDI, indicated that radioactivity reached the bloodstream and that the predominant route of elimination was fecal. The study also included a histologic analysis of the airway which showed the primary effects were congestion of the capillaries, destruction and desquamation of the bronchial epithelium and constriction of the bronchi. Autoradiographic analysis of airway sections was not included. The objectives of the current study were to investigate the distribution of 14C-MDI aerosol in the airways, using histopathologic and autoradiographic methods. Biochemical characterizations of blood components were also undertaken.

EXPERIMENTAL

General Design and Rationale: For these experiments, the MDI was administered to groups of four rats via heads-only inhalation for four hours. Three exposure concentrations were tested, as outlined in Table 1. to address the dose relationship of the biochemical state of the 14C in the bloodstream, to examine the airway distribution through histoautoradiography, and to assess airway damage at a high concentration (6 mg/m3) using unlabeled MDI. The target concentrations of 0.05 and 0.5 and 6 mg/m3 were chosen. Labeled MDI was not used for the 6 mg/m3 experiment due to the difficulty of generating such a concentration with the limited volume of labeled material and the expense involved. Animals for groups 1, 2 and 4 were euthanized immediately following exposure, group 3 was held for 1 week post-exposure to examine clearance, and group 5 was held for 16 hours to examine airway inflammation. Tissues and fluids were collected for analysis from all experimental and six control animals. Endpoint analysis was performed as outlined in Table 1.

Table 1. Protocol Exposure Summary

Exp. #	Target Conc. (mg/m3)	Number of Animals	Duration (hrs)	Exposure Termination	Endpoints
1	0.05	4	4	Im.1	Airway Autoradiography, blood biochemistry, tissue distribution
2	0.5	4	4	Im.	Airway autoradiography, blood biochemistry, tissue distribution
3	0.5	4	4	>/= 72 hr	Clearance, airway autoradiography, blood biochemistry, tissue distribution

4	6	4	4	Im.	Airway histology
5	6	2	4	16 hour	Airway histology and inflammation

1 Im = Immediate

MATERIALS AND METHODS

Chemicals. Monomeric 4,4'-[14C]MDI (28 mCi/mmole) was synthesized by Amersham (Buckinghamshire, England) with the 14C incorporated in the ring. Reverse phase high pressure liquid chromatographic analysis provided by Amersham showed the radiochemical to be 98.9% pure. Derivatization and HPLC analysis of atmospheric samples confirmed the reactivity and purity as 4,4'- MDI (See Quantitation of Isocyanate Concentrations section). Unlabeled 4,4'-MDI was obtained from Bayer, Inc. (Pittsburgh, PA). The unlabeled material was purified by melting point fractionation and was used for the exposure and also as a reference compound to establish the instrument settings for the target 14C exposure concentrations.

Animals. Male, Fischer 344 rats (150 - 200 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN) for the labeled experiments. Taconic Farms was the supplier of the rats for the unlabeled isocyanate experiments. The rats were held in an animal room for at least seven days prior to exposure with food and water provided *ad libitum*. Sixteen rats were used in the 14C study and eight for the nonradioactive experiments. Four exposures were performed with four animals at each concentration and one exposure was done with only two animals. Six additional animals served as controls.

Head-Only Rat 14C-MDI Exposure System. A continuous airflow system was used for all MDI exposures. A four port, glass chamber was constructed with four detachable glass tubes to hold the animals for heads-only exposure. Dental dam collars were fitted around the rats' heads to minimize body contamination and possible dermal absorption. The radioactive MDI was shipped in sealed, glass mini-vials. To generate the MDI condensation aerosol, house air was dried, filtered and delivered over the liquid in the vial once the internal glass septum was broken with a needle. During aerosol generation, the vial was submerged in a constant temperature, paraffin oil bath which was maintained at 86°C for the low concentration and 96°C for the higher concentration exposures. Air flow was controlled by an appropriate flow meter. Choice of flow meters and rate settings, ranging from 4.4 liters/min to 9.2 liters/min for the radioactive experiments was determined using identical, mini-vials containing unlabeled MDI. A 14 gauge needle also penetrated the top of the vial to deliver the material to the condensation arm and into the exposure chamber. The aerosol was drawn into the system by a vacuum pump equipped with a valve and flow meter to regulate the exhaust airflow from the exposure chamber. The exhaust rate was maintained at 12 liters/min for all exposures. For the high concentration, unlabeled MDI exposures, condensation aerosols were generated in a similar system with the primary modification being the use of a glass impinger filled with 0.5 ml of heated MDI.

Characterization of Exposure Atmospheres. Particle size distributions were monitored throughout the exposures through the use of a Marple cascade impactor (Model 290, Andersen Instruments) equipped with an in-line adapter. Mylar substrates were used for particle collection. Each stage was weighed and particle distribution values calculated. For radioactive samples, the filters were placed directly into scintillation fluid for quantitation of total radioactivity.

Quantitation of Isocyanate Concentrations. The quantitation of isocyanate concentrations in the system was performed throughout the 4 hours of exposure by the periodic sampling of the chamber atmosphere using two different assays, as well as the scintillation analysis of assay fluid. One assay used for the determination of isocyanate concentration was the piperazine method (Dow Chemical Co., 1990). The other was the p-nitrobenzoylpropylamine (PNBPA; Regis Chemical Co.) method (Schroeder and Moore, 1985). Both reagents were immobilized on glass fiber filters. The determination of concentration for both procedures involves the derivatization of the reactive isocyanate. Atmospheric samples were drawn through the coated glass fiber filter cassettes at a rate of 2 liters per minute for 15 min periods. The filters were extracted in acetonitrile and an aliquot of the filter extracts was analyzed by reversed phase HPLC. The area of the derivative peaks was quantitated relative to calibration curves.

Collection of Terminal Blood, Body Fluids and Tissues. Animals were euthanized (2 ml Beuthanasia 50 mg/ml, ip) following the experimental timepoints outlined above (Table 1). Terminal blood samples were collected via cardiac puncture and immediately mixed in a Vacutainer tube (Becton Dickinson) containing sodium citrate as an anticoagulant. Two, 200 ml aliquots were placed in glass vials to determine 14C content. To each glass vial, 2.4 ml of NCS tissue solubilizer (Amersham) was added and the suspension was heated at 50°C for 20 min. An aliquot of 0.8 ml of a 20% benzoyl peroxide solution was added followed by an incubation at 50°C for an additional 30 min to decolorize the samples. After cooling to

room temperature, organic scintillant (toluene, 2,5-diphenyloxazole, and 1,4-bis 2-(5-phenyloxazolyl)benzene) was added to bring the final volume to 20 ml. To reduce the level of background radioactivity due to chemiluminescence, the samples were stored in the dark for at least 24 hr before scintillation analysis. Total radioactivity was calculated on both a cpm/ml and microgram equivalents of methylene bisphenyl (MBP) group per milliliter (mgEq MBP/ml) of blood.

The nasal region, trachea, lung, esophagus, stomach and contents, kidney, heart, spleen and liver were collected from each of the exposed animals as well as control animals. The stomach contents were removed from the organ and stored separately. Tissues were either fixed with 10% buffered formalin for histological studies or immediately frozen at -60 ° C until further analyzed.

Tissue Solubilization and Quantitation of Associated Radioactivity. Tissue solubilization and quantitation of 14C content were performed on aliquots of tissue homogenates from the major organs of all exposed and control animals. The fragments were weighed and transferred to glass scintillation vials. Hyamine hydroxide (ICN) was added to a volume six times the total sample weight or a minimal amount of 1 ml and was incubated at 50°C with agitation for 24 hr. Samples were cooled and acidified to pH 6-7 with glacial acetic acid. Scintillant was added and the samples were counted. Results were corrected for background and estimated blood content and the values were normalized on a mgEq MBP per gram tissue basis.

Decalcification and Dissection of the Nasal Cavity. At the time of euthanasia, heads were removed, immersed in 10% buffered formalin, and fixed for a period of 4 weeks. The fixative was removed and replaced with a 30% formic acid solution for decalcification for a period of 3-4 weeks. Four, 0.5 cm thick blocks were cut starting from the anterior nares. The tissues was embedded in paraffin and 5 mm sections were cut from the cephalic side of each block and mounted on glass slides.

Preparation and Fixation of Lungs. The trachea and lung were removed together from the larynx. One 0.2 cm fragment was cut from the mid-section of the trachea and was placed in the fixative. The distal tracheal fragment was left attached to the lung for infusion of approximately 10 ml of 10% buffered formalin. The trachea was tied-off and the whole unit was immersed in the fixative. The mid-section of the trachea was placed on end in the tissue cassette to permit a cross-sectional presentation. The inflated lung was sectioned following an established procedure (Dungworth, *et al.*, 1985) to allow representation of proximal and distal airways and to characterize region-specific distributions of labeling and/or damage.

Analysis of Hematoxylin and Eosin Sections. Slides from each section were deparafinized in xylene and ethanol and then stained with hematoxylin and eosin. Light microscopic examination was performed without prior knowledge of the specimen history. All slides from exposed and control animals were examined and evaluated both in house and by an external pathologist.

Liquid Emulsion Autoradiography. Localization of radioactivity within the tissue was accomplished by liquid emulsion autoradiography. Unstained sections were baked onto glass slides at 45°C for 20 min and deparafinized in xylene, followed by three ethanol washes (100, 95, and 70%, successively). NTB2 autoradiographic emulsion (Kodak) was warmed to 43°C under safelight No. 2 conditions. Slides were sequentially dipped into the emulsion, air-dried for 1 hour and then incubated in light tight storage boxes with hygroscopic calcium sulfate at 4°C for various time periods. Slides were developed in Dektol developer (Kodak) and Kodak fixer (Kodak). Grain deposition was examined by phase-contrast and bright field microscopy.

Ambis 4000 Analysis of Nasal Deposition. Distribution of radioactivity in the nasal region was determined by direct imaging using an AMBIS 4000 system. The paraffin-embedded sections were placed directly into the AMBIS tray and scanned using the 1.6 mm resolution plate. The slides were scanned for 16 hr and region quantitation was performed on the scan results.

Plasma and Cell Isolation. Plasma and cellular blood components were separated by centrifugation of terminal blood samples at 478 x g for 5 min. Plasma was removed and stored at

-60°C. The cellular fractions were stored at 4°C to avoid freeze-fracturing of the cell membranes. To determine the relative distribution of radioactivity in cellular and plasma fractions, 100 ml aliquots of plasma were counted directly in 5 ml Cytoscint ES (ICN), whereas the blood cells were washed with phosphate buffered saline (PBS) to remove extraneous plasma and then solubilized, as described for whole blood, before counting. Distribution of radioactivity in each fraction was calculated as a percentage of the total blood-associated radioactivity.

Molecular Size Fractionation of Plasma. The relative distribution of high and low molecular weight components in plasma was determined by scintillation analysis following separation by molecular size fractionation. This was performed on 0.2 ml aliquots of plasma diluted to 1 ml with PBS, pH 7.4, using Centricon 10 microconcentrators (Amicon). Samples were spun for 40 min at 5000 x g. The retentate was washed with an additional 1 ml of buffer and recentrifuged. Retentates were

recovered by centrifugation at 746 x g for 4 min. Filtrates and retentates were assayed for distribution of radioactivity.

RESULTS

Determination of Reactive Form and Concentration of the Isocyanate Exposure Atmospheres. Chromatographic analysis was performed by Amersham on the 14C MDI which demonstrated a compound purity of 98.9% and a specific activity of 28 mCi/mmole (Figure 1). Reversed phase HPLC analysis was also conducted on the exposure atmospheres following derivatization with PNBPA (Figure 2A) or piperazine (Figure 2B). Fractions were collected across the profiles and counted. A concentration calibration curve was generated with unlabeled 4,4'-MDI and the peak areas were used to determine the concentration of MDI in the radioactive atmospheric samples. Material of the same purity as that used for MDI standard curves was used for the unlabeled MDI exposures. The chromatograms shown are representative of MDI atmospheric profile for all exposure atmospheres. These analyses confirmed the exposure compound purity and concentration. The ability of the radioactive compound to react with the derivatizing chemicals demonstrated that the chemical form used for animal exposure was reactive isocyanate.

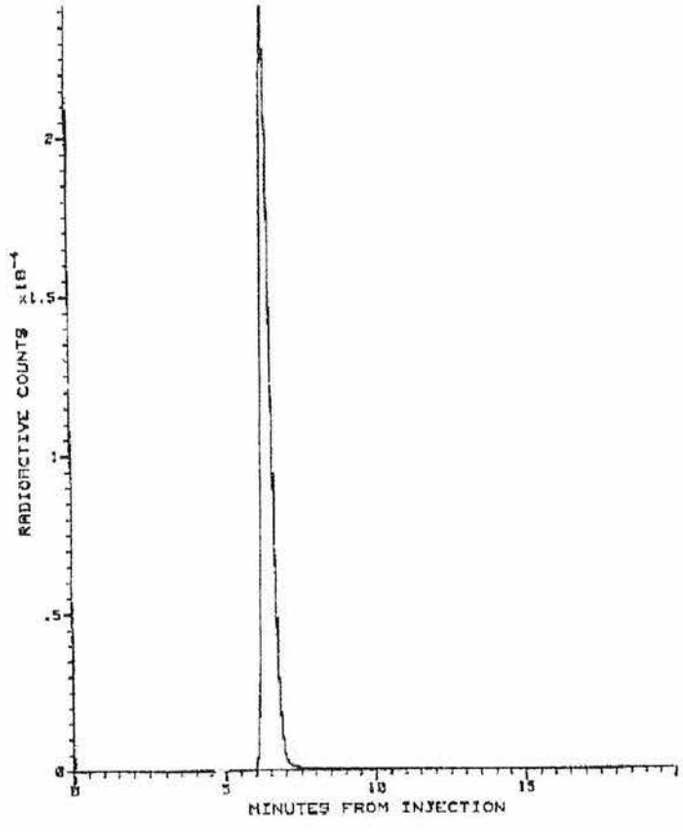

Figure 1: Photocopy of HPLC analysis of synthetic 14C-MDI starting material provided by Amersham Corp. (Data provided by Amersham). High Performance Liquid Chromatography: Amersham: CFQ7144A; 920921A; 4,4'-ME'NEBIS([ring-U-14C]PHE'Isocyanate; QC Number: V21335.

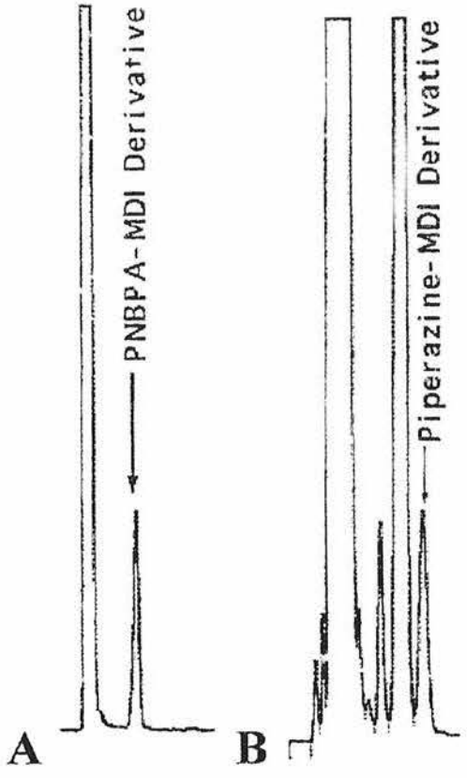

Figure 2: Reverse-phase HPLC Analysis of Derivatized 14C-MDI. (A) Absorbance profile at 254 nm of column effluent following PNBPA derivatization. Derivative peak is indicated by label. Additional peak is excess PNBPA. (B) Absorbance profile at 254 nm of column effluent following piperazine derivatization. Derivative peak is indicated by the label. Additional peaks are excess reagent and by products.

Characterization of Isocyanate Exposure Concentrations. A series of MDI exposures was conducted at three concentrations as given in Table 2. Four methods were used, where appropriate, to monitor the exposure atmospheres: HPLC analysis of PNBPA-derivatized isocyanate, scintillation analysis of the PNBPA trapping filters, HPLC analysis of piperazine-derivatized isocyanate and scintillation analysis of piperazine trapping filters. Table 2 summarizes the compiled data available for each experiment. Multiple samples were collected during the 4 hr exposures at approximately 30 min intervals. The concentration determinations from all methods were averaged. This yielded mean exposure concentrations of 0.052, 0.363 and 0.376 mg/m3 for the 14C MDI experimental series and 6.21 and 5.48 mg/m3 for the unlabeled MDI experiments. Table 3 provides the exposure summary. MDI aerosol particle size was measured during the exposure using a Anderson Marple Cascade Impacter. Atmospheres were drawn through the impacter at a flow rate of 2 liters/minute for one, 60 minute interval during each exposure. Individual stages from the impacter were then analyzed by liquid scintillation counting. The average mean particle diameter for the 3 radioactive experiments was 1.18 μM.

Table 2. MDI Exposure Concentrations

Exp#	PNBPA HPLC (mg/m3 ± S.D.)	PNBPA Radioactivity (mg/m3 ± S.D.)	Piperazine HPLC (mg/m3±S.D.)	Piperazine Radioactivity (mg/m3±S.D.)	Average (mg/m3) ±S.D.
1	0.060 ± 0.002	0.056 ± 0.0002	0.033 ± 0.002	0.059 ± 0.0001	0.052 ± 0.012
2	0.413 ± 0.01	0.373 ± 0.002	0.323 ± 0.016	0.344 ± 0.003	0.363 ± 0.039

3	0.423 ± 0.08	0.393 ± 0.001	0.334 ± 0.017	0.352 ± 0.002	0.376 ± 0.040
4	6.21± 0.49	-	N.D.	-	6.21± 0.49
5	5.48 ± 0.36	-	N.D.	-	5.48 ± 0.36

Table 3: 14C-MDI Exposure Summary

Exp. #	Animal #	Animal Weight (g)	Target MDI Conc. (mg/m3)	Actual MDI Conc. (mg/m3)	Duration of Exposure (hrs)	Time of euthanasia post-exposure
	1	197				
1	2	210	0.05	0.052	4	< 1 hr
	3	206				
	4	216				
	5	189				
2	6	200	0.5	0.363	4	<1 hr
	7	197				
	8	215				
	9	199				
3	10	200	0.5	0.376	4	168 hrs
	11	206				
	12	209				
	13	230				
4	14	222	6.0	6.21	4	<1 hour
	15	216				
	15	228				
5	17	216	6.0	5.48	4	16 hours
	18	207				
	C19	211				
	C20	207				
C	C21	219				
	C22	209				

	C23	204				
	C24	215				

Based on the exposure concentrations, an estimated dose for each exposure group was calculated, assuming 100% retention of the reactive vapor using the following equation: concentration (mg/ml) * time * tidal volume * frequency of respiration. A summary of the estimated doses is given in Table 4.

Table 4. Calculation of Estimated Dose

Concentration (mg/m3)	Concentration (mg/ml)	Time (min)	VT (ml)	f (breaths/min)	Dose (mg)
0.052	5.2 x 10-8	240	1.5	124	2.32 x 10-3
0.363	3.63 x 10-7	240	1.5	139	1.82 x 10-2
0.376	3.76 x 10-7	240	1.5	116	1.53 x 10-2
6.21	6.2 x 10-6	240	1.5	128	2.9 x 10-1
5.48	5.5 x 10-6	240	1.5	136	2.69 x 10-1

Distribution of 14C in Tissues and Fluids of MDI-Exposed Rats. Immediately following each exposure, the experimental group of four rats was euthanized and major organs were collected for scintillation analysis. The results of the digestion of tissue fragments from the esophagus, larynx, liver, kidney and spleen, as well as scintillation analysis of blood, for both exposure concentrations are represented in Figure 3. The inset includes an expanded ordinate axis. Some form of the radioactivity was found associated with all organs analyzed. The specific activity (µgEq MBP/g) of the 14C in all tissues increased with exposure concentration. The percentage of the calculated, estimated dose for each tissue is given in Tables 5 and 6. The radioactive material was cleared from the system as monitored in the urine during the post-exposure period (Figure 4). Despite this clearance, measurable quantities of radioactivity persisted in the organism as shown in the tissue distribution data from exposure group 3 which was held in metabolism cages for one week following exposure (Figure 5).

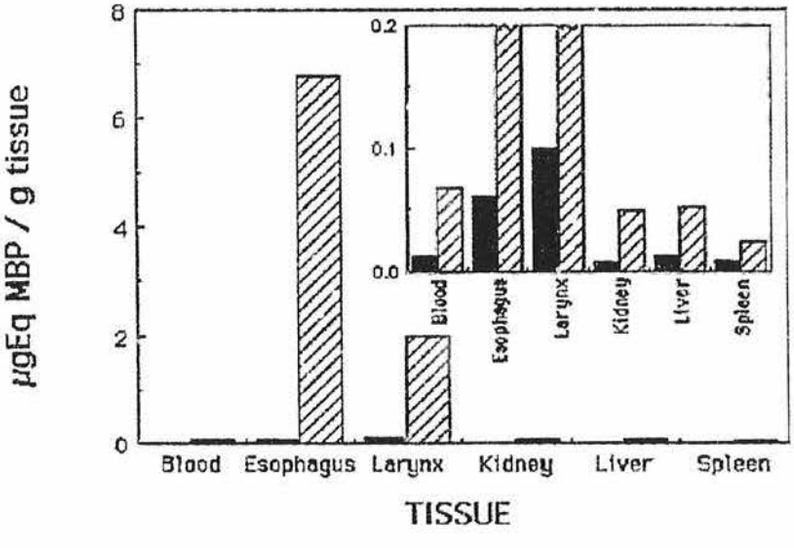

Figure 3: Distribution of Radioactivity in Tissues of Rats Following Inhalation Exposure to 14C-MDI. Tissue distribution of methylene bisphenyl (MBP) group expressed as mgEq/g tissue immediately following14C-MDI inhalation exposure of rats as a function of exposure concentration (0.05 mg/m3 = filled bars; 0.36 mg/m3 = hatched bars). Inset is included as ordinate expansion for clearer representation of selected data.

Table 5. Tissue Distribution Immediately Following a 4 hr MDI

(0.052 mg/m3) Inhalation Exposure

Tissue	Total cpm/ml(g)	Total vol.(weight)	Total cpm	% Dose (100% ret)
blood	2,618 /ml	20 ml	52,360	8.5
larynx	20,349 /g	0.075 g	1,526	0.25
esophagus	13,168 /g	0.06 g	790	0.13
liver	2,443 /g	7.8 g	19,055	3.1
kidney	1,486 /g	0.76 g	1,129	0.13
spleen	1,561 /g	0.5 g	781	0.12

Table 6. Tissue Distribution Immediately Following 4 hr MDI

(0.36 mg/m3) Inhalation Exposure

Tissue	total cpm/ml(g)	total	total cpm	% Dose (100% ret)
blood	14,552 /ml	20 ml	291,040	7.1
larynx	427,804 /g	0.075 g	32,085	0.8
esophagus	1,435,160 /g	0.06 g	86,110	2.1
liver	10,892 /g	7.8 g	84,958	2.1
kidney	10,431 /g	0.76 g	7,928	0.2
spleen	5,050 /g	0.5 g	2,525	0.06

Table 7. Tissue Distribution One Week Following MDI

(0.37 mg/m3) Inhalation Exposure

Tissue	total cpm/ml(g)	total	total cpm	% Dose (100% ret)
blood	1742	20 mL	34840	0.85
larynx	4864	0.11 gm	535	0.01
esophagus	N.D.	0.06 gm	-	-
liver	2046	9.55 gm	19539	0.48
kidney	2932	0.88 gm	2580	0.06
spleen	952	0.54 gm	514	0.01

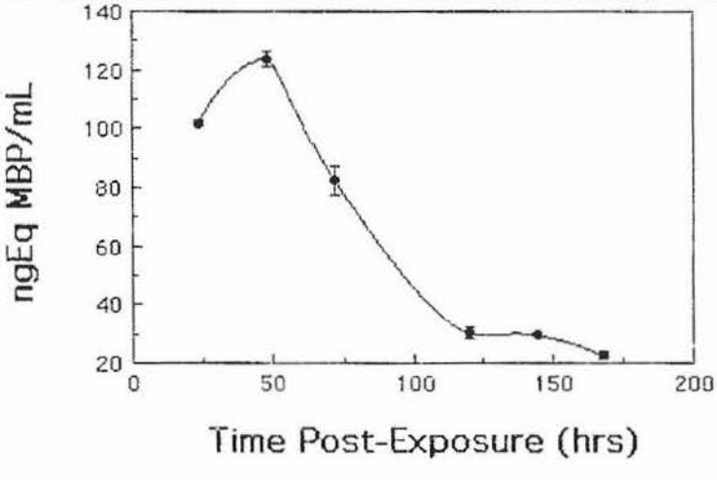

Figure 4: Clearance of 14C from Urine Following 14C-MDI (0.37 mg/m3) Exposure. Aliquots of urine samples collected during the post-exposure period were counted and the 14C quantitation is expressed in ngEq of methylene bisphenyl (MBP)

group per mL of urine.

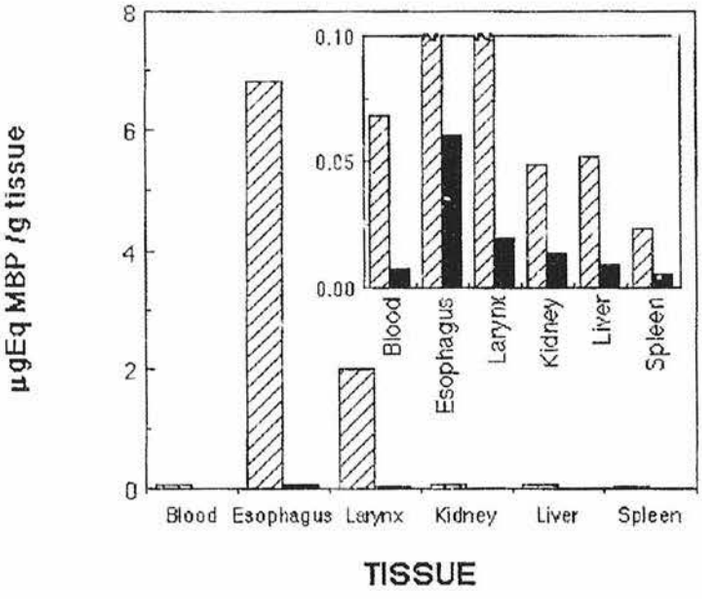

Figure 5: Distribution of Radioactivity in Tissues of Rats One Week Following Inhalation Exposure to 14C-MDI. Tissue distribution of methylene bisphenyl (MBP) group expressed as µgEq MBP/g tissue one week following 14C-MDI inhalation exposure of rats as a function of time post-exposure (immediate = hatched bars; 1 week = filled bars). Inset is included as ordinant expansion for clearer representation of clearance data.

Quantitation of 14C in the Bloodstream of MDI-Exposed Rats. Scintillation analysis of whole blood taken immediately following exposure showed that radioactivity reached the bloodstream. The mgEq MBP value increased in relation to dose for both blood and plasma (Figure 6). Using the calculated total dose, the percentage of the estimated value which was detected in the bloodstream decreased from 8.5 to 7.1 as exposure concentration increased. Even after one week, measurable levels were still observed.

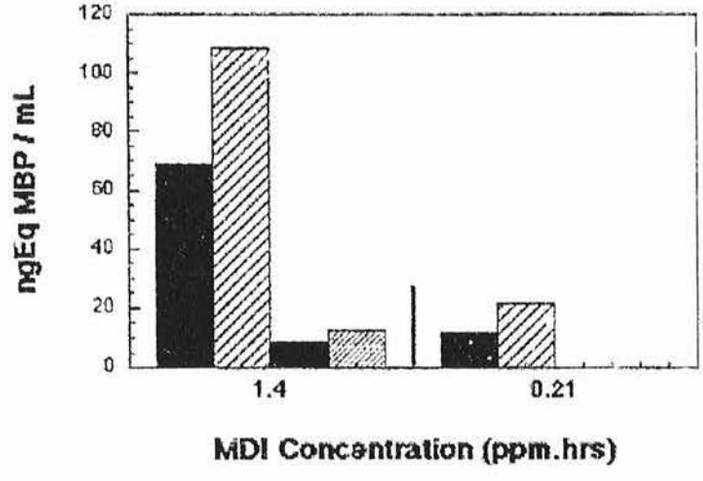

Figure 6. Level of 14C in terminal blood following MDI aerosol exposure. Blood taken immediately = filled bars; plasma taken immediately = hatched bars; blood taken 1 week post-exposure = shaded bars; plasma taken one week post-exposure = narrow hatched bars.

Distribution of 14C in Blood Components of MDI-Exposed Rats. Analysis of radioactivity in whole blood clearly shows that for all exposures, some form of the labeled compound entered the bloodstream. Biochemical analyses of blood samples were performed to characterize the labeled constituents in the blood immediately following the 4 hour exposures. Plasma and cell components were separated and subjected to scintillation analysis. At the concentrations of MDI tested, the majority of radioactivity was found to be plasma-associated; however, the amount of radioactivity in the blood cell pellet fraction was measurable and increased with exposure concentration.

Distribution of Plasma Radioactivity as a Function of Molecular Weight. One of the primary questions regarding the fate of isocyanates in the blood following exposure is whether there are low molecular weight compounds (e.g. MDI, oligoureas or metabolites) and/or high molecular weight adducts. To address this question, plasma samples were subjected to molecular fractionation using Centricon-10 microconcentrators which separate the high molecular weight (>10kDa) conjugates from low molecular weight (<10kDa) conjugates and metabolites. The distribution of radioactivity in the retentate (>10 kDa) and filtrate (<10 kDa) fractions was determined by scintillation analysis (Table 8). The results show that the predominant form (93-101%) of the radioactivity in the plasma immediately following a 4 hour exposure was conjugated material greater than 10 kDa in molecular weight.

Table 8: Molecular Sieve Fractionation of Plasma

	Analysis of Fraction > 10kDa		Analysis of Fraction < 10kDa	
Exposure	(Retentate Fraction)		(Filtrate Fraction)	
#	Total ngEq	% of original	Total ngEq	% of original
1	4.6	93	ND	ND
2	18.3	92	0.6	3
3	3.6	101	0.07	2

In addition, plasma was subjected to SDS-PAGE analysis and the resulting gel was cut into equal size pieces, digested and each fraction analyzed by scintillation counting. The resulting electropherogram (Figure 8) shows the predominant band of radioactivity centered around the molecular weight of serum albumin (68 kDa) with minor amounts of radioactivity distributed along the rest of the gel. For comparison, a parallel analysis was performed with plasma from 14C-TDI exposed rats. Previous work from a 14C-TDI study (Kennedy et al., 1994) has shown the radioactive label to be associated with a 70 kDa protein that co-migrates with rat serum albumin.

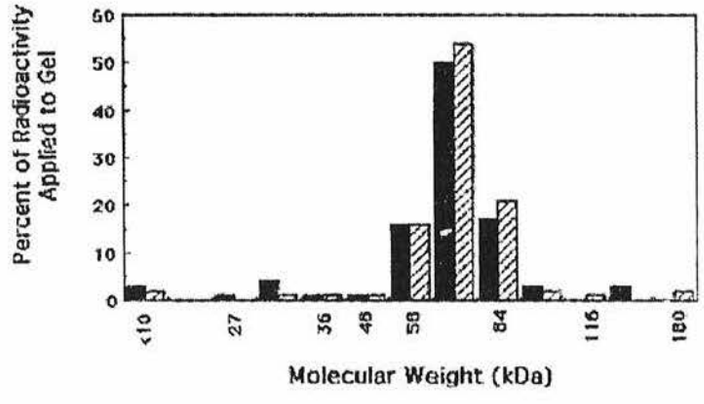

Figure 7. SDS-PAGE analysis of plasma from 14C-MDI exposed rats. Plasma from 14C-MDI exposed rats were subjected to SDS-PAGE and resulting gel was cut into equal fragments, digested and analyzed by scintillation counting. For comparison, plasma from 14C-TDI exposed rats (Kennedy et al., 1994) was analyzed on the same gel. The solid bars represent the TDI results while the hatched bars represent the results from the MDI analysis.

Analysis of Airway Deposition and Damage. One of the primary objectives of this study was to analyze the distribution and fate of the inhaled 14C-MDI aerosols in the airway. Nasal and lung tissues were fixed and processed for light microscopic analysis. Hematoxylin and eosin stained sections were analyzed for cellular damage associated with exposure. Minimal damage was observed and was limited to focal areas of epithelial desquamation. Emulsion deposition was observed on the epithelial surface in all regions of the airway(Figure 8).

Figure 8. Deposition of 14C in Rat Airways Following 14C-MDI Inhalation Exposure. Tissue autoradiography of (A) trachea and (B) lung tissue sections taken from a rat exposed to 0.5 mg/m3 MDI for four hours. Sections are viewed at 230X.

Due to the higher level of radioactivity in the nasal section, direct imaging was performed on the AMBIS 4000 system. The resultant distribution of label is given in Figure 9. The two dimensional representation illustrates the intensity of labeling on the nasal septum and lighter, focal areas in the turbinates. The histologic analysis of airway sections following a 4 hour exposure to 6 mg/m3 unlabeled MDI was performed to asses acute tissue damage as compared to setions from control animals. In all regions of the airways, no significant alterations were detected in the exposed animal specimens (Figures 10 and 11). When compared to the unexposed controls, there was no destruction of the normal cell architecture, no fluid accumulation and no evidence of inflammatory cell migration

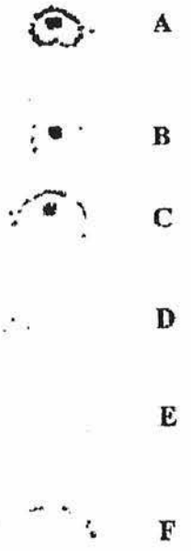

Figure 9: AMBIS 4000 Analysis of 14C Distribution in Nasal Tissues. From A to F are sections taken from the anterior to posterior regions of rat nasal region following 0.37 mg/m3 14C-MDI exposure. The spot in the center of sections A-C indicate intense labeling of the nasal septum, while the ring that loses intensity from anterior to posterior represents labeling of the turbinates.

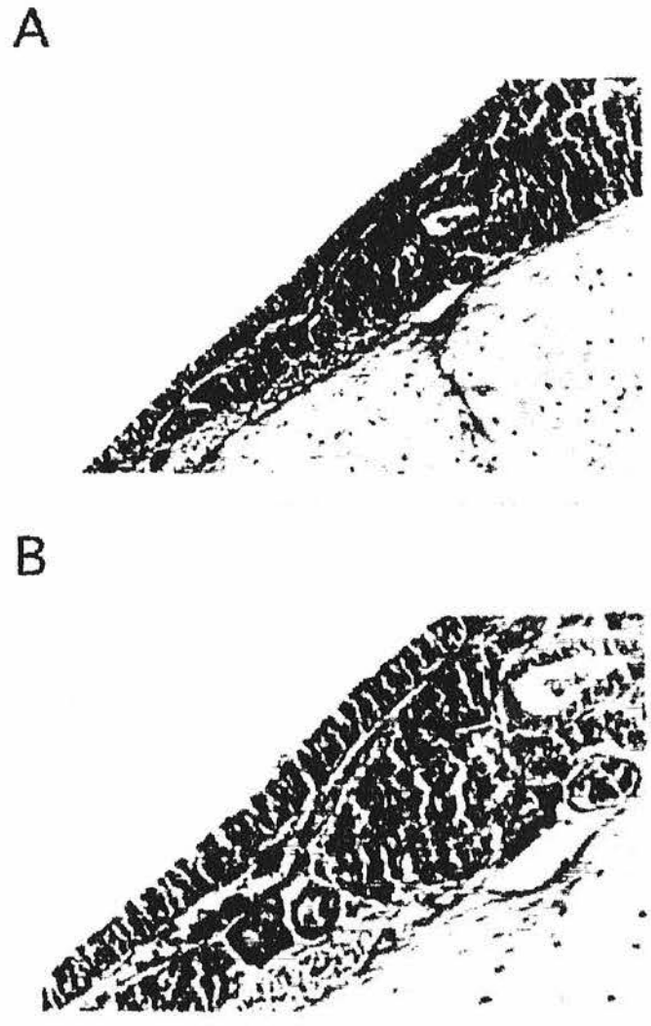

Figure 10. Histology of Nasal Region following inhalation of 6 mg/m3 MDI for 4 hours. Nasal section from rat exposed to 6 mg/m3 MDI for four hours. (A) 115X, (B) 230X.

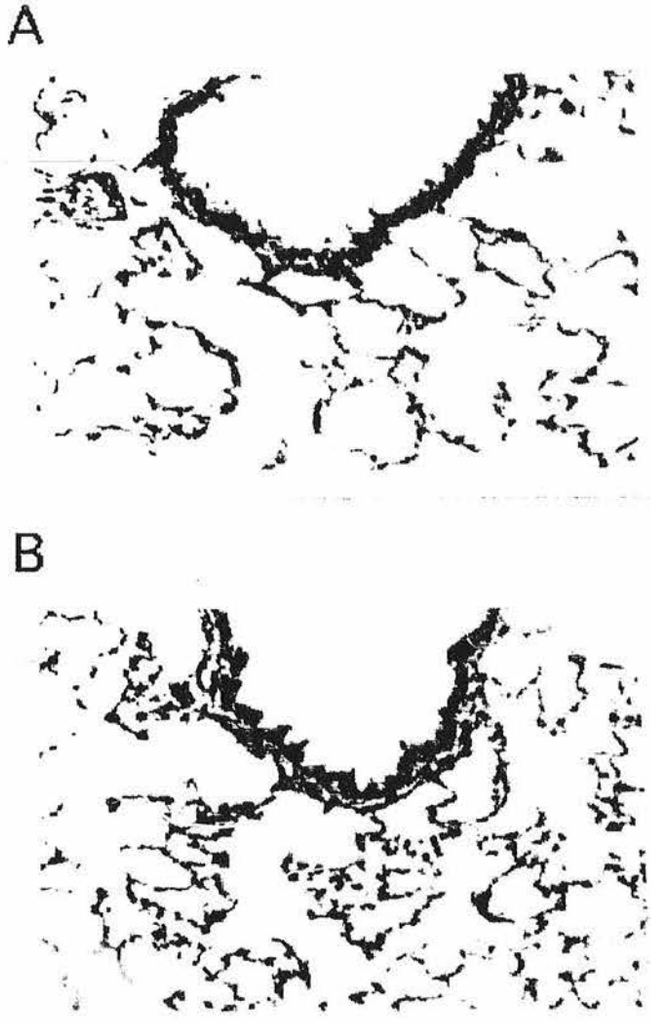

Figure 11. Histology of Lung Tissue following inhalation of 6 mg/m3 unlabeled MDI for 4 hours. Two separate lung sections from a rat exposed to 6 mg/m3 MDI for four hours. Both sections are viewed at 230X magnification.

SUMMARY

Acute four hour exposures of rats to three separate concentrations of MDI were performed. At 0.052 and 0.36 mg/m3 MDI concentrations, the isocyanate was ring labeled with 14C, while in the 6 mg/m3 MDI concentration the exposure was performed with unlabeled MDI. Aerosol concentrations were stable throughout all exposures as monitored by two independent HPLC methods. Mean aerosol particle diameters were equivalent between experiments at 1.18 μM.

Appearance of 14C following the exposures was concentrated in the airway tissues and the blood. The level of radioactivity was directly related to the exposure concentration and decreased gradually during the post-exposure period. A minimum plateau of radioactivity appeared to be reached at 120 hours post-exposure. 95-100% of the plasma radioactivity was recovered in the retentate fractions (>10 kDa). SDS PAGE analysis of the plasma fraction greater than 10 kDa showed a single, labeled component of 70 kDa.

Histological examination of the airway tissues was conduction on samples from all of the exposures. Sections from all airway regions were processed for hemotoxalin and eosin staining and emulsion autoradioagraphy was performed on tissues from 14C exposed rats. At the 0.052 and 0.36 mg/m3 MDI concentrations, tissue autoradiography shows that the label was

associated with the surface of the epithelial layer. Minimal inflammatory response was evident in the post-exposure sample for both the 0.36 and 6 mg/m3 MDI exposure concentrations. Cell damage was limited to focal areas of epithelial injury at mg/m3 MDI.

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November 23, 1998

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Dear Sir or Madam:

We herewith submit a copy of the following recently completed health and safety study: "Investigation of the Chemistry of TDI in Biological Materials".

Name of chemical substance:	benzene, 2,4-diisocyanato-1-methyl
Common name	2,4-toluene diisocyanate
Chemical Abstracts Service number:	584-84-9
Abbreviation:	2,4-TDI

Name of chemical substance:	benzene, 1,3-diisocyanatomethyl-
Common name	generic toluene diisocyanate
Chemical Abstracts Service number:	26471-62-5
Abbreviation:	2,4-TDI and 2,6-TDI (mixture)

Authors	A.L. Kennedy and W.E. Brown

The International Isocyanate Institute (III) project identification number (11320) has been marked on the title page of the report. Please refer to the III identification number in any communication regarding this study. The enclosed reports do not contain any Confidential Business Information.

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